

Urinary Markers for Exposures to Alkylating or Nitrosating Agents

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Investigation of urinary markers as indices of endogenous nitrosation and of gastric cancer etiology has been a major focus of our work. As part of this effort, studies have been carried out on a Colombian population at high risk for gastric cancer. In this group, nitrosoproline excretion was highly correlated with nitrate excretion in the subpopulation with advanced gastric pathology, but not in control subpopulations with more normal stomachs. Neither urinary 7-methylguanine nor 3-methyladenine was strongly related to gastric pathology or to urinary nitrate or nitrosoproline levels. More recently, as evidence has accumulated concerning the importance of nitric oxide as a cellular messenger, we have begun research toward developing markers for the presence of nitric oxide and for endogenous nitrosation via this compound. Nitric oxide is formed from arginine by activated endothelial cells as a messenger for vasodilation. We have shown that prolonged exercise leads to increased urinary nitrate and that when ¹⁵N-arginine is ingested by humans, ¹⁵N-nitrate levels increase in 24-hr urine collections. Nitrosohydroxyethylglycine and 3-nitrotyrosine were evaluated as indices for the formation of *N*-nitrosomorpholine and for the nitration of protein, respectively, under experimental conditions (e.g., immunostimulation) expected to enhance nitric oxide formation. Nitrotyrosine has not proved useful as a biomarker for nitration/nitrosation reactions in immunostimulated rats. Immunostimulation of rats following administration of morpholine led to increases in urinary nitrate and nitrosohydroxyethylglycine. This procedure, however, would not be appropriate for humans due to the toxicity of morpholine and the carcinogenicity of *N*-nitrosomorpholine.

Introduction

During the past several years we have made a major effort toward validating the use of protein adducts as dosimeters for individual exposure to carcinogens. Depending on the proteins used, most often albumin or hemoglobin, the adducts reflect the average exposure over the weeks or months preceding the analysis. Analysis of urine for evidence of exposure to xenobiotics, on the other hand, typically reveals exposure during the previous day or two, making these analyses complementary to protein adduct analyses. Urine analysis has advantages (e.g., large amounts of sample are easily collected by noninvasive means) and drawbacks (e.g., excreted metabolites may represent detoxication pathways and there may be very low levels of the compounds of interest). In addition to these general characteristics, a major focus in the investigation of urinary exposure markers has been the assessment of DNA damage via quantitation of DNA repair products, especially alkylated bases, which are excreted in urine.

The most common application in this context has been the measurement of urinary nitrosoamino acids to estimate levels of gastric nitrosation. This technique was first reported by Ohshima and Bartsch (1), who proposed the use of *N*-nitrosoproline (NPRO), a noncarcinogenic *N*-nitroso compound formed from proline, which was naturally present in the human body and which could be safely administered in sufficient amounts so that the *N*-nitroso derivative could be easily quantitated in the urine. This method has been used by other researchers who have confirmed that nitrosamines are formed endogenously (2-5). In human studies, urinary NPRO levels increase when nitrate and L-proline doses are given, and NPRO excretion returns to baseline levels (14-30 nmole/day) when a large dose (1 g) of ascorbic acid is given along with the L-proline. *N*-Nitrosoproline has also been used as a biomarker in larger studies of populations at high risk for cancer. Lu and co-workers, for example, used levels of four urinary *N*-nitrosoamino acids, including NPRO, as an index of individual exposure to *N*-nitroso compounds or their precursors ingested and/or formed endogenously in high- and low-risk areas for esophageal cancer in northern China (6). When the subjects followed their normal dietary patterns, NPRO and nitrate levels in 24-hr urine samples were significantly higher in the high-risk area than in the low-risk area, thus supporting the hypothesis that exposure to *N*-nitroso compounds contributed to the higher risk. *N*-Nitrosothiazolidine carboxylic acid (NTCA), first identified by Ohshima and co-workers (7), is also used as

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an indicator of endogenous nitrosation in both rats and humans (3,8,9). The precursor, thiazolidine-4-carboxylic acid (TCA) is a condensation product of cysteine and formaldehyde, and its formation may represent a detoxification pathway for formaldehyde (10). *N*-Nitrosothiazolidine carboxylic acid is similar to NPRO in that it is nonvolatile, nontoxic, nonmutagenic, and not metabolized. In addition, NTCA is formed about 1000-fold faster than NPRO (8,11) and is therefore potentially more sensitive than NPRO as an indicator of nitrosation.

These methods are based on the analyses of nonmetabolized *N*-nitroso compounds; the test compounds are thus noncarcinogenic and excreted quantitatively. In some specific circumstances, however, it has been possible to identify carcinogenic nitroso compounds in human urine. A recent example of this was the report by Tricker et al. that several nitrosamines including *N*-nitrosodimethylamine, *N*-nitrosodiethylamine, *N*-nitrosopiperidine, and *N*-nitrosopyrrolidine were detected in the urine of paraplegics (12).

In addition to markers for endogenous nitrosation, there has been a growing interest in alkylated nucleic acid bases as urinary markers for DNA damage by carcinogen metabolites. Although 7-methylguanine had been mentioned in this context as early as 1967 by Craddock and Magee (13), this class of compounds was not practical as markers for human exposure to alkylating agents until sufficiently sensitive techniques such as GC-MS became routinely available. Farmer and Shuker and their co-workers have now shown that it is indeed possible to monitor both 7-methylguanine and 3-methyladenine in human populations (14-18).

We, along with many other groups, have been interested both in the biochemistry of endogenous nitrosation and in the possible involvement of *N*-nitroso compounds (especially when endogenously formed) in human cancer. The remainder of this report summarizes some of our approaches to these problems via quantitation of various related substances in urine by methods developed by us or adapted from others.

Analytical Methods

Nitrosoproline was quantitated as the methyl derivative by GC-thermal energy analyzer (TEA) (1) and nitrate by an automated method using the Griess reagent (19). Incorporation of ¹⁵N into urinary nitrate was measured by GC-MS after conversion of the nitrate to nitrobenzene (19). 3-Methyladenine and 7-methylguanine were extracted from urine and purified with XAD-2 resin followed by bonded-phase extraction column chromatography with carboxylic acid cartridges (20). They were then converted to the *tert*-butyldimethylsilyl derivatives and quantitated by electron-ionization GC-MS with the trideuterio analogs as internal standards. Nitrotyrosine (NTyr) and *N*-nitrosohydroxyethylglycine were analyzed as the *tert*-butyldimethylsilyl derivatives by GC-TEA or GC-MS with either positive chemical ionization or negative ion chemical ionization for NTyr and electron ionization for *N*-nitrosohydroxyethylglycine (21).

Results and Discussion

Urinary Nitrosation and DNA-Damage Markers for Gastric Cancer Risk

Our projects of longest duration in this area are focused on the risk factors leading to high rates of gastric cancer in the high-

lands of Colombia, the biochemistry of endogenous nitrate synthesis and endogenous nitrosation, and the possible relationships, if any, among them. In the Colombian gastric cancer study, a hypothesis evolved in which nitrate in the drinking water was reduced to nitrite by salivary bacteria or by gastric bacteria populating the achlorhydric stomach environment, which accompanied the developing gastric pathology (22,23). The nitrite was then presumably converted to nitrosating agents which in turn converted compounds in the stomach with reactive -NH bonds to carcinogenic *N*-nitroso compounds. This hypothesis was strengthened when 4-chloro-6-methoxyindole, which forms a direct-acting mutagen when nitrosated (24), was isolated from fava beans.

Because urinary *N*-nitrosoproline levels could be used as indices of gastric nitrosation (1), and because urinary 3-methyladenine (17,25), and to a lesser extent 7-methylguanine (13,14), reflected methylation of DNA, we evaluated these compounds, as well as nitrate, in the urine of a large group of people from the high-gastric cancer risk area of Colombia. The objectives were to determine if the levels of these compounds varied among the various levels of gastric pathology and if any of the indices varied in concert. We were particularly interested in whether increased levels of nitrate were reflected in increased levels of NPRO and 3-methyladenine. Increased NPRO would suggest enhanced gastric nitrosation and increased 3-methyladenine might reflect increased formation of alkylating intermediates, e.g., *N*-nitrosodimethylamine. (3-Methyladenine-d₃ arises directly from endogenously formed NDMA-d₆ following administration of dimethylamine-d₆ and nitrite to ferrets; see below.)

A group of 160 randomly chosen people was assembled from a high-risk area in the Nariño region of Colombia. Information about smoking and alcohol consumption was obtained by interview. On the basis of histological examination of gastric biopsy specimens, the participants were assigned to one of the following groups: normal gastric pathology, superficial gastritis, chronic atrophic gastritis, chronic atrophic gastritis with intestinal metaplasia, or dysplasia. Twenty-four-hour urine samples from each participant were analyzed for nitrate, *N*-nitrosoproline, 3-methyladenine, and 7-methylguanine. Linear regressions were carried out on all combinations of the data sets. The two-sample *t*-test was used to determine differences between smokers and nonsmokers and for drinkers and nondrinkers.

There was a weak but significant correlation ($r = 0.297$, $p = 0.0001$, $n = 160$) between NPRO and nitrate for the entire population which strengthened for the combined groups with metaplasia and dysplasia ($r = 0.60$, $p = 0.0001$, $n = 37$). There were no other notable correlations except a weak relationship between NPRO and 3-methyladenine ($r = 0.19$, $p = 0.02$, $n = 156$). Overall excretions of all four compounds were the same, (i.e., no statistically significant differences), for all gastric pathology groups, but 3-methyladenine and 7-methylguanine levels were higher for smokers than for nonsmokers.

3-Methyladenine from Endogenously Formed *N*-Nitrosodimethylamine

As noted above, the appearance above dietary contributions (18) of 3-methyladenine in urine is an indicator for methylation

Table 1. 3-Methyladenine from endogenous formation of *N*-nitrosodimethylamine.

	Treatment	3-Methyladenine-d ₃ , ng ^a
Animal 1	None	ND
	DMA-d ₆	30
	DMA-d ₆ + nitrite	150
Animal 2	None	ND
	DMA-d ₆	17
	DMA-d ₆ + nitrite	60

Abbreviations: DMA, dimethylamine; ND, none detected.

^aValues for trideuterated 3-methyladenine represent the total amount excreted in one 24-hr period after dosing.

of DNA followed by excision repair. As part of a study of endogenous formation of NDMA in ferrets, NDMA or its precursor, dimethylamine, were labeled with stable isotopes to distinguish 3-methyladenine arising from metabolism of the nitrosamine from that arising from other metabolic processes. The objectives were to demonstrate methylation of DNA via endogenously formed NDMA and to compare DNA methylation with methylation of hemoglobin. Hexadeuterio-NDMA or hexadeuterio-dimethylamine and sodium nitrite were administered to ferrets by various routes; urine was then analyzed for [³H₃C]3-methyladenine as an index of DNA methylation. Labeled 3-methyladenine was observed at levels corresponding to prior nitrosation yields of 0.05–0.1% following administration of labeled dimethylamine and nitrite (Table 1). GC–MS analysis confirmed the presence of intact methyl groups, indicating that methylation occurred via metabolism of endogenously formed NDMA rather than by incorporation of dimethylamine into the one-carbon pool. This was supported by related experiments with ¹⁴C-labeled NDMA in which the animal was treated with 4-methylpyrazole, an inhibitor of NDMA metabolism (27,28), prior to administration of NDMA. In this case, no labeled 3-methyladenine was observed. Methylated hemoglobin, quantitated via GC–MS analysis of labeled methanol released on hydrolysis (29), was observed in parallel with the 3-methyladenine in all of these experiments.

Markers for Endogenous Formation of Nitric Oxide

As the Colombian project developed, and with the discoveries that several cell types, including bacteria (30,31), macrophages (32,33), and endothelial cells (34), could produce nitric oxide under certain conditions (Fig. 1), some major changes in thinking about endogenous nitrosation, previously believed to occur primarily in the acidic stomach, became necessary. Reaction of nitric oxide with oxygen produces the nitrosating agents N₂O₃ and N₂O₄, the former being an especially effective nitrosating agent at non-acidic pH (Fig. 1). These processes raised the possibility that gastric nitrosation might occur at the elevated gastric pH often observed in the Colombian high-risk group as well as at other locations in the body. It thus became of interest to develop, if possible, dosimeters for the formation *in vivo* of nitric oxide or its oxidation products and for the related nitrosation of precarcinogens at physiological pH.

In the most straightforward example of this, prolonged intense exercise, expected to produce nitric oxide as a vasodilator in response to increased oxygen demand, led to increased urinary

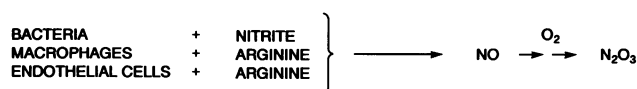


FIGURE 1. Nitric oxide from various cell types. *E. coli* grown anaerobically in the presence of nitrate will reduce nitrite to nitric oxide (30,31). Macrophages stimulated with *E. coli* lipopolysaccharide and/or γ -interferon (32,33) and endothelial cells stimulated with bradykinin (34) oxidize arginine through a complex pathway to produce nitric oxide.

nitrate excretion in two human volunteers (21,35). In another experiment, ¹⁵N-arginine was ingested by humans and urinary nitrate was analyzed by GC–MS for incorporation of label. ¹⁵N-Nitrate levels were elevated during the subsequent 24-hr, demonstrating that the arginine–nitric oxide pathway was valid in humans (36).

3-Nitrotyrosine

Recently, Ohshima and co-workers (37) suggested that the formation of nitrotyrosine might be useful as an index for endogenous nitrosation and potentially for nitric oxide or its oxidation products; although not a urinary marker, our experiments with NTyr were done concurrently with *N*-nitroso(2-hydroxyethyl)-glycine and nitrate analyses for stimulated nitric oxide production. This compound is especially appealing for human use because the precursor is naturally present in the body. Nitrotyrosine is readily formed in peptides and proteins after incubation with nitrite or tetranitromethane *in vitro* and in rats following treatment with tetranitromethane. Metabolites of an oral dose of NTyr are excreted in urine: about 44% as 3-nitro-4-hydroxyphenylacetic acid (NHPA) and about 5% as 3-nitro-4-hydroxyphenylactic acid (NHPL); NHPA has also been detected in human urine (37), although its origin is unknown.

Formation of NTyr *in vivo* from biologically relevant nitrosating agents such as NO₂ and NO_x has not yet been demonstrated. In experiments with rats, no increase in NTyr was detected above background levels in globin or plasma protein following administration of nitrite, although NTyr was observed in both plasma protein and globin following treatment of blood with nitric oxide. This was also true for animals treated with lipopolysaccharide (LPS) or with *Salmonella* endotoxin, although both of these treatments resulted in elevated levels of urinary nitrate. Blood from rats treated with tetranitromethane (TNM) contained NTyr in plasma proteins as well as in globin. Tetranitromethane is well known as an effective nitrating agent for proteins. The reaction of nitrite with proteins, however, is strongly dependent on pH; reaction at pH 3.5 is over 2000-fold faster than reaction at pH 7. Formation of NTyr *in vivo* may also be hampered by the rapid reaction of nitrite or nitric oxide with hemoglobin.

N-Nitroso-(2-hydroxyethyl)-glycine

N-Nitroso(2-hydroxyethyl)glycine (NHEG) was identified by Hecht and Young (38) as the major urinary metabolite of *N*-nitrosomorpholine (NMOR) in which the *N*-nitroso group was retained; about 40% of an NMOR dose is excreted as NHEG. The amine precursor, morpholine, is readily nitrosated

in vitro (39), by macrophages in culture (40), and in the stomach (41). *N*-nitrosomorpholine itself, however, is rapidly metabolized in the whole animal and is, therefore, of little direct use as an indicator of endogenous nitrosation.

N-Nitroso(2-hydroxyethyl)glycine has been used as a biomarker for endogenous nitrosation in LPS-stimulated rats (35). Lipopolysaccharide-induced immunostimulation is followed by an increase in urinary excretion of NHEG, almost certainly due to enhanced nitrosation of morpholine. After LPS treatment, average urinary NHEG rose from about 24 pmole/day to about 160 pmole/day, along with a 25-fold increase (from about 5 μ mole/day to about 130 μ mole/day) in urinary nitrate. *N*-Nitroso(2-hydroxyethyl)glycine levels were not increased following administration of either nitrate or morpholine (except in the latter case for that arising from a small amount of NMOR present as an impurity in the morpholine); the nitrosating agent appears to be the limiting reagent and to arise directly from nitric oxide rather than from the recycling of nitrate to nitrosating agents.

Alkylated adenines other than 3-methyladenine may be useful dosimeters in specific situations, and 3-methyladenine, as noted in the Colombian study, may be useful in situations where dietary variations are minimal or can be controlled. Although many urinary markers have been useful for elucidating mechanisms in animal experiments, the most informative urinary indexes for humans have been the simplest, e.g., nitrate and nitrosoproline.

This manuscript was presented at the Conference on Biomonitoring and Susceptibility Markers in Human Cancer: Applications in Molecular Epidemiology and Risk Assessment that was held in Kailua-Kona, Hawaii, 26 October–1 November 1991.

We are grateful for support from grant no. CA26731 and training grant ES07020 from the National Institutes of Health, grant no. I-S10-RR01901 from the Department of Health and Human Services Program, and grant no. ES02109 from the National Institute of Environmental Health Sciences.

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